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Effect of malic acid supplementation on rumen fermentation, digestibility and methanogenesis in wheat straw sorghum based total mixed diets *in vitro*

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ABSTRACT

An *in vitro* incubation system was used to evaluate effect of increasing concentration of malic acid at 0, 5, 10 and 15 mM in wheat straw sorghum diets of different roughage (R) and concentrate (C) ratios i.e. high fiber (80R:20C), medium fiber (50R:50C) and low fiber (20R:80C) diets on different rumen fermentation, total gas and methane production and gas kinetics parameters. All the treatment combinations were arranged in 4 × 3 factorial designs with 3 replicates. Feed samples (200 mg) were incubated in 100 ml calibrated glass syringes with 30 ml mixed rumen suspension for 24 h (methane and fermentation parameters) and 96 h for gas kinetics. *In vitro* dry matter digestibility (IVDMD) was significantly higher at 5 and 10 mM malic acid in almost all type of diets. A significantly increasing trend of partition factor and microbial biomass (mg) content was seen with increasing concentration of malic acid. Methane (mM/g DM) reduction was 47 to 65% in wheat straw sorghum diets. Significant reduction in ammonia- N concentration was noticed, however decreasing trend in protozoa number was seen in all type of diets but differences among diets remained nonsignificant. Potential gas production and rate constant increased in all dietary treatment combinations. The malic acid was significantly able to modify the rumen fermentation parameters which contributed towards reducing the methane production and the effect was variable depending upon different roughage and concentrate ratios.

Key words: Malic acid, Gas kinetics, Partitioning factor, Microbial biomass, Methane production

Malic acid is an intermediate in the succinate propionate pathway of ruminal bacteria (Castillo *et al.* 2004). *In vitro*, malic acid has stimulated lactate utilization (Nisbet and Martin, 1991), increased concentrations of propionate, total volatile fatty acids, increased pH (Martin and Streeter 1995, Carro and Ranilla 2003), decreased methane production and lactate concentration (Carro and Ranilla 2003), and increased digestibility of dry matter, organic matter, neutral detergent fiber and hemicellulose (Carro *et al.* 1999). The recent goal of ruminant microbiologists and nutritionists is to manipulate the ruminal microbial ecosystem to improve the feed conversion efficiency and reduce methane production. Much of the research in the past decades has focused on the effects of antimicrobial compounds on ruminal fermentation mainly on ionophores and antibiotics (Russell and Strobel 1989). These compounds seem to inhibit hydrogen-producing microorganisms and Gram-positive lactate-producing bacteria such as *Streptococcus bovis* (Russell 1987, Russell and Strobel 1989). Use of organic acids in ruminants diets opens an alternative to antibiotics and ionophores with interesting results by decreasing methane and change in fermentation can – stimulate the growth of the prominent ruminal bacterium, *Selenomonas ruminantium*; favorably alter the mixed ruminal microorganism fermentation; and improve the performance of feedlot steers (Martin and Streeter 1995, Callaway and Martin 1996). The present study was conducted to evaluate the effect of increasing concentration of malic acid on rumen fermentation, total gas and methane production and gas kinetics parameters.

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MATERIALS AND METHODS

Experimental design: To evaluate the effect of different doses of malic acid, three diets were prepared by taking different roughages and concentrates in the ratio of 80: 20 (HFD, high fiber diet), 50: 50 (MFD, medium fiber diet) and 20: 80 (LFD, low fiber diet). The roughage part composed of wheat straw (70 parts) and sorghum (30 parts) and the concentrate part composed of maize (33 parts), GNC (21 parts), mustard cake (12 parts), wheat bran (20 parts), deoiled rice bran (11 parts), mineral mixture (2 parts) and salt (1 parts). Malic acid was added in incubation medium to achieve final concentration of 0, 5, 10 and 15 mM. All the treatment

combinations were arranged in 4 × 3 factorial designs with 3 replicates. A set was incubated devoid of substrate with and with out malic acid which served as blanks for particular treatment and control.

Preparation of inoculums: Rumen liquor was collected from a fistulated male buffalo maintained on a standard diet (60 parts roughage: 40 parts concentrate) before morning feeding into an insulated flask and brought into the laboratory. The rumen liquor filtered through four layers of muslin cloth and then the required amount of filtered rumen liquor used as a source of inoculum.

In vitro *gas production:* The substrate was milled to pass through 1 mm sieve and 200±10 mg was weighed in glass syringes of 100 ml capacity. The incubation medium was prepared as described by Menke and Steingass (1988). The 30 ml incubation medium was dispensed anaerobically in each syringe. Syringes were incubated at 39±0.5° C for 24 h. Before incubation, malic acid solution was injected as per the dose by small syringe into 100 ml syringes individually. Plungers of syringes applied with petroleum jelly for smooth movement and stop any leakage. Syringes were closed using clamps and were incubated for 24 h in case of *in vitro* dry matter digestibility (IVDMD), total volatile fatty acids (TVFAs), individual volatile fatty acids (IVFAs) and methane, while incubated for 96 h (in sequential incubation for 0, 1, 2, 3, 6, 9, 12, 24, 30, 36, 48, 60, 72 and 96 h) in case of gas kinetics study.

Total gas production and methane estimation: After 24 h incubation, total gas production was estimated by the displacement of piston during incubation. The gas produced due to fermentation of substrate was calculated by subtracting gas produced in blank syringe (containing no substrate, but only the inoculum and buffer) from total gas produced in the syringe containing substrate and inoculum and buffer. For methane estimation, representative gas was sampled from the headspace of syringe in an airtight syringe and injected into gas chromatograph equipped with flame ionization detector (FID) and stainless steel column packed with Porapak-Q. The gas flow rates for nitrogen, hydrogen and air were 30, 30 and 300 ml/min, respectively. Temperature of injector oven, column oven and detector were 40, 50 and 50° C, respectively. A 50/50 mixture of methane and carbon dioxide was used as a standard.

Partitioning factor and microbial biomass yield: The PF is calculated as the ratio of substrate truly degraded *in vitro* (mg) to the volume of gas (ml) produced by it. Substrate provides important information about partitioning of fermentation products. The MBM yield was calculated by using the degradability of substrate and gas volume and stoichiometrical factor as suggested by Blummel *et al*. (1997):

Microbial mass = Substrate truly degraded-(gas volume × stoichiometrical factor)

where the stoichiometrical factor used was 2.25.

Gas production kinetics: The total gas production kinetics was carried out in different treatment combinations incubated as per procedure mentioned above for different intervals i.e. 0, 1, 2, 3, 6, 9, 12, 24, 30, 36, 48, 60, 72 and 96 h. The potential gas production and rate of gas production was calculated by fitting the modified equation given by Orskov and McDonald (1979).

Estimation of ammonia nitrogen: The supernatant of each syringe including that of blank was used for NH_3-N estimation. Supernatant (5 ml) was mixed with 1 N NaOH (2 ml) and steam passed on this using nitrogen analyzer and the NH_3 evolved was collected in boric acid solution having mixed indicator and titrated against N / 100 H_2SO_4.

Total volatile fatty acid (TVFA) and individual volatile fatty acid (IVFA) estimation: TVFA concentration (mmol/ 100 ml) in the supernatant was estimated according to Barnett and Reid (1957). For the IVFA estimation, at the end of incubation (24h) 1 ml of the supernatant was treated with 25% meta-phosphoric (4 ml) and kept for 3–4 h at ambient temperature (Erwin *et al.* 1961). Thereafter, it was centrifuged at 3000 rpm for 10 min and clear supernatant was collected and stored at –20°C until analyzed. IVFA estimated using gas chromatograph equipped with flame ionization detector (FID) and stainless steel column (length 4'; o.d ¼''; i.d 3 mm) packed with Chromosorb 101. Temperature of injection port, column and detector was set at 200, 180 and 210°C, respectively. The flow rate of carrier gas (nitrogen) through the column was 40 ml/ min; and the flow rate of hydrogen and air through FID was 30 and 300 ml/ min, respectively. Sample (2 ml) was injected through the injection port using syringe (10 ml). Individual VFA's of the samples were identified on the basis of their retention time and their concentration (mM) and calculated by comparing the retention time as well as the peak area of standards after deducting the corresponding blank values.

Calculation of reducing equivalents and contribution of reductive acetogenesis to overall fermentation: According to Van Navel and Demeyer (1995) recoveries of reducing equivalents (2H) in the *in vitro* fermentations were calculated from the stoichiometry of rumen fermentation as follows, where M is methane, A is acetate, P is propionate, B is butyrate, and V is valerate:

$$\%\ H_2 \text{ recovery} = 2H \text{ accepted}/2H \text{ released} \times 100,$$

where 2H released = 2A + P + 4B + 3V and 2H accepted = 2P + 2B + 4V + 4M.

The contribution of reductive acetogenesis to overall rumen fermentations was calculated using the method of Faichney *et al.* (1999) as follows:

Reductive acetogenesis = (1.8A – 1.1P + 1.6B – 1.3V – 4 M)/5.8A + 11.6B,

where M is methane, A is acetate, P is propionate, and B is butyrate.

Protozoa counting: For protozoal count one milliliter of the fermentation fluid was diluted with 1 ml of formalin (18.5% formaldehyde) and 3–4 drops of brilliant green and

then incubated for 24 h at room temperature. The stained protozoa were diluted (if needed) and counted by haemocytometer as per Dehority (1984).

In vitro *true DM degradability:* To estimate true DM degradability of feed sample of each syringe containing residues after incubation was estimated as per Van Soest *et al.* (1991).

Proximate analyses and cell wall constituents: The proximate analysis of substrate was carried out as per AOAC (1995). The NDF content of substrate was determined according to Van Soest *et al.* (1991) and ADF as per AOAC (1990) index no. 973. 18.

Statistical analyses: Experimental data of different parameters were analyzed in 4 × 3 factorial randomized block design with 3 replicates for analysis of variance as per Snedecor and Cochran (1968). The effects of different diet and doses of malic acid compared with control were tested using the factorial arrangement in complete randomized block design in OPSTAT statistical software (Sheoran 2010). When the overall *F*-test was significant, differences between means and the control were declared significant at $P \leq 0.05$ using the Fisher's-least-significant-difference (critical difference).

RESULTS AND DISCUSSION

The chemical composition of different high, medium and low fiber wheat straw sorghum diets was presented in Table 1. The crude protein (CP) and ether extract (EE) contents were increased with decreasing level of roughage in different diets. CP content ranges from 115.5 to 196.3 g/kg and NDF content ranges from 279.0 to 575.2 g/kg of feed dry matter in wheat straw sorghum diets. The effect of malic acid could be influenced by the composition of diet, therefore, in *in vitro* study 3 diets mainly low, medium and high fibre wheat straw sorghum diet were used.

Results of *in vitro* study indicated that in all cases the pH relatively stable at near range in wheat straw sorghum diets (6.75–6.87). There was a nonsignificant difference in pH of all types of wheat straw sorghum diets. Earlier reports conclude that a greater response in methane production occurred with free acids compared with sodium salts.

Table 1. Chemical composition of wheat straw sorghum diets

Diets	Particulars (g/kg on DM basis)						
	OM	CP	EE	NDF	ADF	HC	TA
HFD(80R: 20C)	893.4	115.5	18.5	575.2	391.4	183.8	106.6
MFD(50R: 50C)	900.0	178.1	22.6	422.6	290.1	132.5	100.0
LFD(20R: 80C)	901.9	196.3	35.2	279.0	192.2	86.8	98.1

OM, organic matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber; HC, hemicellulose; TA, total ash; HFD, high fiber diet; MFD, medium fiber diet; LFD, low fiber diet; R, roughage; C, concentrate.* roughage part composed of wheat straw (70 parts) and sorghum (30 parts).

Table 2. Malic acid supplementation effect on pH, digestibility, microbial biomass, protozoa and ammonia-N production

Wheat straw sorghum diet	Dose (mM)	Parameter					
		pH	DDM (mg)	PF (mg)	MBM (mg)	NH_3-N (mg/100 ml)	Protozoa ($\times 10^4$ml $^{-1}$)
HFD(80R: 20C)	0	6.83	131.00	3.36	41.75	11.67	2.00
	5	6.81	133.67	3.16	38.42	10.27	1.50
	10	6.75	127.00	3.55	46.00	10.27	1.17
	15	6.79	121.67	5.07	63.92	8.87	1.33
MFD(50R: 50C)	0	6.87	148.00	3.22	35.38	14.93	0.83
	5	6.87	148.33	3.27	46.34	13.53	0.70
	10	6.83	147.67	3.41	50.44	12.60	1.00
	15	6.75	151.67	4.12	68.04	12.13	1.33
LFD(20R: 80C)	0	6.85	153.33	3.19	44.96	16.80	1.33
	5	6.82	158.33	3.37	52.21	17.27	1.40
	10	6.80	157.33	3.55	57.59	16.80	1.50
	15	6.75	157.33	3.82	58.75	16.10	1.33
SEM	Diet(D)	NS	1.55	NS	NS	0.26	NS
	Treatment(T)	NS	NS	0.19	3.33	0.30	NS
	D*T	NS	NS	NS	NS	NS	NS

HFD, high fiber diet; MFD, medium fiber diet; LFD, low fiber diet; R, roughage; C, concentrate; DDM, digestible dry matter (mg); PF, partition factor (mg TDMD / ml gas); MBM, microbial biomass (mg); NH_3-N, ammonia nitrogen.SEM, standard error of means.

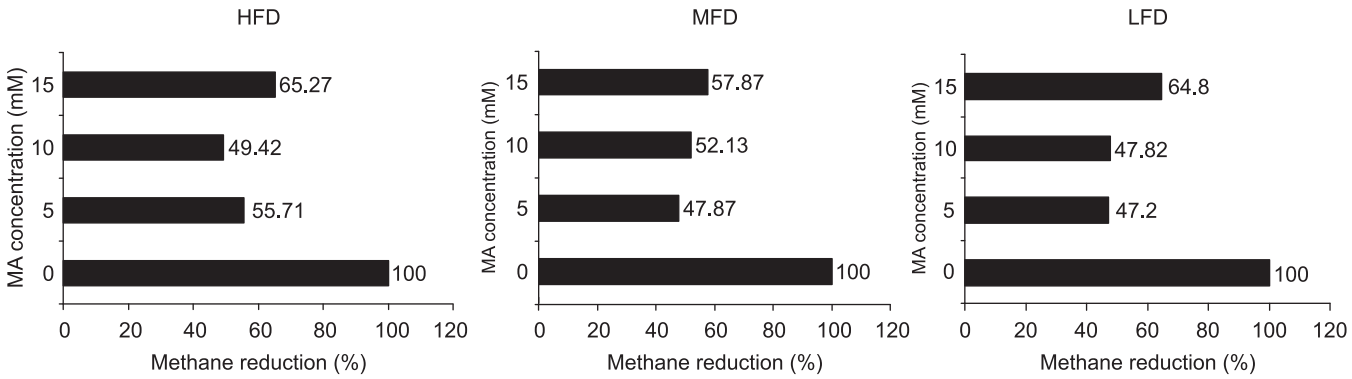

Fig.1. Methane reduction percentage in different wheat straw sorghum diets supplemented with malic acid.

Although the addition of free acids was associated with a decrease in the pH of the medium, the change was very small and the resultant pH remained in the range optimal for CH_4 formation (Russell 1998). Unless solubility or rate of dissolution was affected, it is unclear why the free acids were more effective.

The addition of malic acid in wheat straw sorghum diets increased digestible dry matter content (DDM/ 200 mg of incubated feed DM) and effect was variable related to diet and concentration of malic acid (Table 2). Results of DM digestibility of wheat straw sorghum diets found to be not affected by malic acid supplementation. The results were non-significant among different treatments and diets due to different concentrations of malic acid. These results are in agreement with the results previously reported by other workers (Carro *et al.* 1999, Gomez *et al.* 2005 and Sniffen *et al.* 2006) with diets of varying composition. In most of the studies addition of malate increased dry matter digestibility. The apparent DM degradation was increased in batch cultures by between 10 and 16% by the addition of different organic acids including fumarate, 2-oxoglutarate, malate and tartrate. In addition, it was also proposed that cellulolytic organisms benefit from the presence of methanogens or other H_2-utilizing bacteria because of interspecies H_2 transfer (Wolin *et al.* 1997). Therefore, if H_2 removal increased by the supplementation of propionate precursors, may further increase or stimulate the growth of fibrolytic bacteria (Lo´pez *et al.* 1999) and result in increased cellulose digestion (Asanuma and Hino 2000).

In the present experiment, on wheat straw sorghum diets, both partition factor (PF) and microbial biomass production (MBM mg) increased significantly according to increasing concentration of malic acid (Table 2). The highest increase in PF was 50, 27 and 19% and highest increase in MBM (mg) was 53, 92 and 30% at 15 mM malic acid concentration in HFD, MFD and LFD, respectively. The PF of the diets is an index of microbial biomass synthesis efficiency (Bl¨ummel *et al.* 1997) and the diet formulation to achieve

Table 3. Malic acid supplementation effect on total gas and methane production

Wheat straw sorghum diet	Dose(mM)	Parameters			
		Gas produced (mM)	CH_4 (mM/g DM)	TVFA/CH_4 ratio	A/P ratio
HFD(80R: 20C)	0	7.94	1.73	24.15	2.45
	5	8.48	1.24	39.14	2.45
	10	7.21	0.86	63.00	2.15
	15	5.14	0.41	194.03	1.78
MFD(50R: 50C)	0	9.28	2.46	19.81	2.84
	5	9.08	1.40	35.31	2.35
	10	8.68	1.14	52.23	2.15
	15	7.44	0.70	94.89	1.85
HFD(20R: 80C)	0	9.64	2.59	21.65	2.76
	5	9.44	1.47	38.54	2.27
	10	8.88	1.23	46.01	2.18
	15	8.28	0.73	98.71	1.95
SEM	Diet(D)	0.24	0.04	NS	NS
	Treatment(T)	0.28	0.05	12.87	0.06
	D*T	NS	0.09	NS	NS

TVFA/CH_4 ratio, total volatile fatty acid and methane ratio; A/P ratio, acetate propionate ratio.

higher PF would mean aiming for higher microbial biomass synthesis *in vivo* (Bl¨ummel *et al.* 2003). Factor which increases organic matter degradability, such as the soluble unfermented organic matter, or that decreases gas production, such as the degraded protein, contribute to higher partitioning factor (Bl¨ummel *et al.* 2003). In the present experiment, gas production values were higher ($P < 0{\cdot}05$) for malate-supplemented diets than control, but when gas production values were corrected for the amount of gas released from malate fermentation, the gas production values were reduced in treatment combinations as also reported by Tejido *et al.* (2005).

Total gas produced (mM) during 24h *in vitro* incubation due to malic acid supplementation is presented in Table 3. In high fiber wheat straw sorghum diet total gas reduction was highest i.e. 35.3% at 15 mM dose and it was 9.2% at 10 mM dose whereas total gas was increased by 6.8% at 5 mM dose. Reduction in total gas production was 2.2, 6.5 and 19.8% at 5, 10 and 15 mM malic acid concentration in medium fiber wheat straw sorghum diet. In low fiber diet (Fig. 1), total gas reduction was 2.1, 7.9 and 14.1% at 5, 10 and 15 mM malic acid doses, respectively. Methane (mM/g DM) reduction was highest i.e. 76.30, 71.54 and 71.81% at 15 mM dose in high, medium and low fiber wheat straw sorghum diets, respectively (Table 3). Its reduction at 5 mM dose was 28.32, 43.09 and 43.24% and at 10 mM dose was 50.29, 53.66 and 52.51% in high, medium and low fiber diet, respectively. In all 3 types of wheat straw sorghum diets methane reduction increased as the concentration of malic acid increased from 5 mM to 15 mM. TVFA to methane ratio in diet increased 62.07, 160.87 and 703.44% in HFD; 78.24, 163.65 and 379% in MFD; 78.01, 112.52 and 355.94% in LFD at 5 mM, 10 mM and 15 mM dose, respectively (Table 3). Acetate to propionate ratio in the same diet increased 12.2% and 27.3% at 10 and 15 mM malic acid dose in HFD, as it was not affected at 5 mM dose. In MFD, A/P ratio was 17.3, 24.3 and 34.9% and in LFD it was 17.8, 21.0 and 29.3% at 5, 10 and 15 mM concentration.

Methane (CH_4) production in rumen is affected by many factors, such as the type of diet and the rumen pH etc. Further, it was found that only few numbers of methanogens were detected in the rumen in concentrate fed animals than in the rumen of forage fed animals (Demeyer and Fievez 2000). The conversion of starchy and cellulosic feed to acetate, propionate and butyrate in the rumen results in an overall net release of reducing power. Much of this is used by methanogenic archaea to reduce CO_2 to CH_4, but H can also be used as a substrate in malate reduction (Russell and Wallace 1997). Malic acid has been suggested to act as an electron sink for *S. ruminantium* (Nisbet and Martin 1991). Rather than having a toxic effect on H_2 producing bacteria like ionophores, malate addition seems to stimulate succinate and / or propionate production by *S. ruminantium,* resulting in a decrease in availability of H_2 to methanogenic bacteria. A significant methane reduction was observed in different treatment groups further indicated the positive correlation of above theories proposed by different workers.

Results of total volatile fatty acid (TVFA) production inferred that TVFA increased significantly with the supplementation in all type of wheat straw sorghum diets in this study. Highest increase was noticed at 15 mM malic acid concentration in comparison with other malic acid concentration i.e. 0, 5 and 10 mM (Table 4). The TVFA concentration was 41.7, 48.7, 51.0 and 60.7 mM in HFD;

Table 4. Supplementation effect of malic acid on rumen fermentation in wheat straw sorghum diets

Wheat straw sorghum diet	Dose (mM)	Parameters					
		TVFA (mM)	Acetate (mM)	Propionate (mM)	Butyrate (mM)	% 2H recovery	Acetogenesis (%)
HFD (80R: 20C)	0	41.7	25.9	11.0	4.8	80.60	3.74
	5	48.7	31.5	12.9	4.4	53.34	14.66
	10	51.0	31.9	14.8	4.3	59.29	12.52
	15	60.7	35.8	20.1	4.8	55.71	14.44
MFD (50R: 50C)	0	48.7	32.3	11.4	5.1	84.97	1.97
	5	49.3	31.1	13.2	5.0	65.52	9.80
	10	59.5	31.3	14.9	5.1	65.94	9.79
	15	63.0	37.0	20.0	6.0	61.47	11.85
LFD (20R: 80C)	0	56.0	36.1	13.1	6.8	81.19	3.44
	5	56.3	34.5	15.2	6.6	63.96	10.41
	10	56.3	29.5	13.8	5.7	70.08	8.03
	15	67.0	41.0	21.0	5.0	56.92	13.75
SEM	Diet (D)	1.42	NS	NS	0.21	1.31	0.53
	Treatment (T)	1.64	1.34	0.47	NS	1.51	0.61
	D*T	NS	NS	NS	NS	NS	NS

TVFA, total volatile fatty acids (mM); H, hydrogen; SEM, standard error of means.

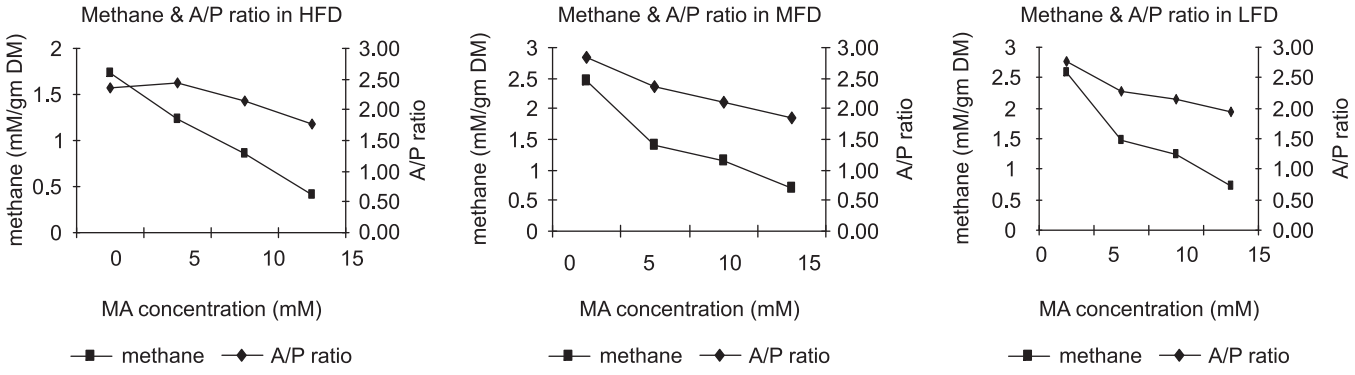

Fig. 2. Methane and A/P ratio in different wheat straw sorghum diets supplemented with malic acid. HFD, high fiber diet; MFD, medium fiber diet; LFD, low fiber diet

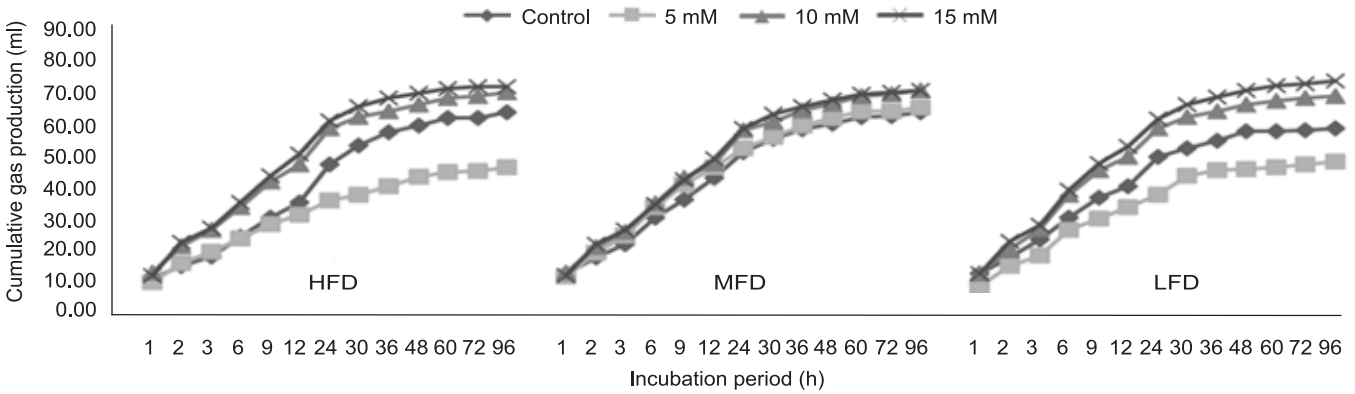

Fig. 3. Malic acid supplementation effect on cumulative gas production in high, medium and low fiber wheat straw sorghum diets during 96h *in vitro* incubations. HFD, high fiber diet; MFD, medium fiber diet; LFD, low fiber diet

48.7, 49.3, 59.5 and 63.0 mM in MFD and 56.0, 56.3, 56.3 and 67.0 mM in LFD at 0, 5, 10 and 15 mM malic acid concentration, respectively. The percent increase of TVFA at 15 mM malic acid concentration was 45%, 29% and 19% in HFD, MFD and LFD, respectively. The increased VFA production observed with all substrates is in accordance with previously reported results (Russell and Van Soest 1984). Increases in the production of both acetate and propionate were reported when batch cultures containing different cereal grains were supplemented with 7 and 10 mM / l malate (Carro and Ranilla 2003), although other authors found increases in propionate production without significant changes in the production of acetate when malate (4 to 12 mM/l) was added to batch cultures containing cracked maize or hay (Martin and Streeter 1995, Martin 2004). These inconsistent results by different authors may be due to different substrate and experiment conditions; however in the present study the increase in acetic acid and propionic acid was observed in most of the cases. Almost an increasing trend in acetate concentration was noticed in HFD (38%) and MFD (14%). But in case of LFD, acetate concentration reduced significantly at 5 and 10 mM concentration while it was increased up to 13% at 15 mM malic acid concentration. Propionate concentration increased significantly according to increasing doses of malic acid (Fig. 2). The values of propionate concentration at 0, 5, 10 and 15 mM malic acid concentration were 11.0, 12.9, 14.8 and 20.1 mM; 11.4, 13.2, 14.9 and 20.0 mM and 13.1, 15.2, 13.8 and 21.0 mM in HFD, MFD and LFD, respectively. The increase in propionate percentage at 5, 10 and 15 mM malic acid concentration was 17.27, 34.55 and 82.73%; 15.79, 30.70 and 75.44% and 16.03, 5.34 and 60.31% in HFD, MFD and LFD, respectively.

In all wheat straw sorghum diets a significant treatment effect was seen at different concentrations of malic acid on ammonia- N concentration. The decrease in ammonia-N concentration was according to increasing concentration of malic acid and the decrease was highest at 15 mM (Table 2). It was 23.99, 18.75 and 4.17% in high, medium and low fibre diets at 15 mM malic acid concentration. Sanson and Stallcup (1984) also reported *in vitro* studies that dl-malate favorably alters ruminal nitrogen fermentation pattern in sorghum based diets. But recently, Gomez *et al.* (2005) and Sniffen *et al.* (2006) observed that there were no differences among treatments for NH_3-N, non-ammonia-N, non-microbial N. The inconsistent results observed with supplemental malic acid can partially be explained by the

Table 5. Effect of malic acid on gas kinetics (96h) in wheat straw sorghum diets

Regression model – Orskov and McDonald (1979) without lag									Equation:– F=b*(1-exp (-c*x))			
Diet type	HFD (80R: 20C)				MFD (50R: 50C)				LFD (20R: 80C)			
Dose (mM)	0	5 mM	10 mM	15 mM	0	5 mM	10 mM	15 mM	0	5 mM	10 mM	15 mM
B	69.726	45.900	75.130	78.844	67.873	68.654	75.244	76.339	62.679	49.515	73.935	79.259
C	0.064	0.109	0.103	0.105	0.095	0.113	0.105	0.105	0.108	0.108	0.121	0.119
R^2	0.992	0.948	0.982	0.986	0.984	0.977	0.980	0.982	0.981	0.978	0.987	0.985

b, Potential gas production (ml); c, gas production rate constant (ml/h); R^2, regression coefficient.

diet or substrate incubated and experimental conditions. They also reported that an increase in microbial N production with the addition the malate to semi-continuous and continuous culture systems, respectively. There was a numerical increase in efficiency of microbial synthesis (unit microbial N / unit DM digested) with the addition of malate. A nonsignificant but apparently decreasing trend in protozoa number was seen in all type of wheat straw sorghum diets.

Results related to gas kinetics of wheat straw sorghum diet due to malic acid supplementation are presented in Table 5. It was observed from the results that potential gas production (b) was increased due to addition of malic acid in wheat straw sorghum diets. The increase was highest at 15 mM malic acid concentration and the increase in b values was 13%, 12% and 26% in HFD, MFD and LFD, respectively. Similarly the gas production rate constant (c) also increased in almost in all treatment combinations in comparison to control in high, medium and low fiber wheat straw sorghum diets.

The ability of dicarboxylic acids to stimulate ruminal fermentation may be associated with differences in pH, although these may be small, and to the removal of H_2. The hydrogen recovery and reductive acetogenesis data calculated from VFA data are shown in Table 4 for wheat straw sorghum diets. The % hydrogen recovery increases with increasing portion of concentrate in the diet. In the present experiment, significant increase in %H recovery was observed in LFD. The mean %H recovery for wheat straw sorghum based HFD, MFD and LFD was 62.24, 69.48 and 68.04% respectively. Average hydrogen recovery values (53.34–84.97%) in wheat straw sorghum diets suggest the presence of hydrogenotrophic acetogenesis (Marounek and Rada 1998). In methanogenic cultures of the rumen content such recoveries are seldom lower than 80% (Demeyer *et al.* 1989). Several authors demonstrated a competition for hydrogen for methanogenic archaea and hydrogenotrophic acetogenic bacteria. In energetic terms, competition for hydrogen should favor methanogens rather than acetogens (ΔG_0'= -131 and -95 kJ.mol^{-1}, respectively). Reductive acetogenesis made a significant contribution (1.97 to 14.66%) to overall ruminal fermentation in wheat straw sorghum diet.

Overall it was inferred that malic acid had a beneficial effect on methane reduction by modifying rumen fermentation pattern. The *in vitro* dry matter digestibility (IVDMD) was significantly (P< 0.05) higher at 5 and 10 mM malic acid in almost all type of diets. A significantly increasing (P<0.05) trend of partition factor and microbial bio mass (mg) content was seen with increasing concentration of malic acid. Methane (mM/g DM) reduction was 47 to 65% in wheat straw sorghum diets. A shift in rumen fermentation pattern is towards more propionic acid production as a result ruminal hydrogen availability for methane production is decreased. Significant (P<0.05) reduction in ammonia- N concentration was noticed in wheat straw sorghum diets with increasing concentration of malic acid, however decreasing trend in protozoa number was seen in all type of wheat straw sorghum diets but differences among diets remained non-significant. Potential gas production increased in wheat straw sorghum diets. Gas production rate constant increased in all dietary treatment combinations (Fig. 3).

It was concluded from the study that malic acid was significantly able to modify the rumen fermentation parameters which contributed towards reducing the methane production and the effect was variable depending upon different roughage and concentrate ratios. More studies with diets of different composition are required to assess the dietary conditions that influence the effectiveness of malate and its long term effect under *in viv*o conditions.

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